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Original research article

Isolation and Identification of Citrobacter Species and their Antibiotic Susceptibility with Special Reference to Extended Spectrum Beta-Lactamase

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Abstract

Background: Citrobacter species are frequent nosocomial pathogens , a local or systemic breach in the host defences can allow them to cause a range of infections which includeurinary tract infections , neonatal sepsis , pulmonary infections , meningitis and blood stream infections.*Citrobacter* species are emerging as important nosocomial pathogens . *Citrobacter* infections are associated with high mortality rate.

Objectives: To determine their antibiotic susceptibility pattern. To detect extended spectrum beta lactamase producing species.

Material and Methods: Various clinical samples received in the central lab of IGIMS Hospital , Patna Bihar. Identification will be done by using standard microbiological techniques. Antibiotic susceptibility test will be performed by Kirby-Bauer Disc diffusion method. Test strains will be pre-incubated in peptone water at 37^{o}C at an optical density of 0.5McFarland standard. This suspension will be used to inoculate strains onto Muller Hinton agar plate by swabbing them with a sterile cotton swab and performing culture as recommended by clinical and laboratory standards institute . The antibiotic discs used for sensitivity testing will be obtained from Hi-Media , India , which will be used to study the sensitivity pattern.

Conclusion: The magnitude of *Citrobacter* infections has increased over time considering its potentialto cause nosocomial infections and the growing numbers of immunocompromised patients in hospitals. *C.koseri* and *C.freundii* being the commonest species isolated. Theyare usually isolated from patients with wound infections, urinary tract infections , respiratory infections and bacteremia. The emergence of drug resistance among the *Citrobacter* is noteworthy**.**

Keywords: *Citrobacter spp, Citrobacter* infections, UTI, Antibiotic SusceptibilityPattern, ESBL.

Introduction

These bacilli are commonly distributed in soil, sewage, water& food. The importance of this species lies in theirassociation with serious nosocomial infections and high degree resistance to commonantimicrobial agents used for the treatment of various infections[1] . *Citrobacter species*and *Serratia marcescens* constituted 1-2% of nosocomial bloodstream, cardiovascular and ear, nose and throat infections[1]. Although *Citrobacter species* are infrequent nosocomialpathogens, local or systemic breaches of host defenses can allow them to cause a rangeof infections. These include urinary tract infections, neonatal sepsis, brain abscess , meningitis , bloodstream infections , intra-abdominal sepsis , and pneumonia[2]. The *Citrobacter species* are emerging as important nosocomial pathogens. Antibiotic resistance is emerging in the isolates of *Enterobacteriaceae* and in other gram negative bacilli in many parts of the world and this is a major threat to the successful treatment ofinfections in hospitals[3]. The *Citrobacter* infections are associated with a high mortality rate. They are often resistant to the routinely used antibiotics, especially the extended spectrum cephalosporins, due to the overexpression of chromosomal beta lactamases[3]. Urinary tract infection (UTI) continues to be the commonest nosocomial infection according for approximately 40% of all hospital acquired infections and it is one of the most important causes of morbidity and mortality[4]. Urinary tract infection (UTI) is the third most common infection experienced by humans after respiratory and gastro-intestinal infections[4]. *Citrobacter* isolates were found to be the third most common organism causing UTI in hospitalized patients after Escherichia coli and Klebsiella species accounting to 9.4% of all isolates. It is a present challenge to the clinical microbiologist because of their increased occurrence in nosocomial infection[4]. extended-spectrum beta-lactamases (ESBLs) have become more and more prevalent in species characterized by inducible class C cephalosporinase (AmpC) such as *Citrobacter freundii and Serratia marcescens*, which frequently segregate mutants with high-level constitutive production of AmpC enzymes[5]. . Although the resistance rates of *Serratia sp and Citrobacter sp* to third-generation cephalosporins areconsiderably high in India, studies on the resistance mechanisms to extended-spectrum cephalosporins among these species have been rarely performed here[5]. Increasing drug resistance due to empirical treatment in UTI needs regular monitoring ofthe antibiotic susceptibility of uropathogens in a particular area. Various factors such as the type of UTI (complicated or uncomplicated), gender, age, and previous history of antibiotic therapy or instrumentation of each UTI patient should also be considered to find out the correct global data on susceptibility[6]. Isolated pathogen frequency and antimicrobial resistant rates can vary dramatically even within the same country. To ensure appropriate therapy current knowledge of the pathogens that cause UTI in an areaand their susceptibility pattern is mandatory[7] .

Objectives

To isolate and identify *Citrobacter species* from various clinical samples, study the antibiotic susceptibility of *Citrobacter species* isolated, detect the occurance of extended spectrum beta-lactamase producers in the *Citrobacter species* isolated.

Review of Literature

Citrobacter species belong to a group of facultative anaerobic, Gram negative bacilli within the family *Enterobacteriacea*. They are frequently found in water, soil, food and intestines of animals and humans. Previously recognised as environmental contaminantsor colonizes with low virulence. They are now known to cause wide spectrum of infections involving urinary tract, liver, biliary tract, peritoneum, intestines, bone, respiratory tract,

endocardium, wounds, soft tissue, meninges and the bloodstream. *Citrobacter species* account for 0.8% of all Gram negative infections and represent approximately 3-6% of the isolates of *Enterobacteriaceae*[8]. *Citrobacter* organisms are inhabitants of the intestinal tract. The most common clinical manifestation in patients as a result of infection occurs in the urinary tract. However, additional infections, including septicemias, meningitis, brain abscesses, and neurologic complications, have been associated with *Citrobacter spp*. Transmission is typically person to person. The clinically relevant members of the *Enterobacteriaceae* can be considered as two groups: the opportunistic pathogens and the intestinal pathogens. The opportunistic pathogens most commonly include Citrobacter spp., Enterobacter spp., Klebsiella spp.,Proteus spp., Serratia spp., and a variety of other organisms. The *Citrobacter* isolates in study conducted by Gandham Pavani et al. showed 100% of the isolates were sensitive to Amikacin , 56% of the isolates were sensitive to Norfloxacin , 56% to ciprofloxacin , 20% to Cotrimaxazole , 14% to Nalidixic Acid and 12% to Ampicillin[9] Resistance to b-lactams among *Citrobacter spp*. is common and mediated through production of b-lactamases[10]. *C. freundii* is considered inherently resistant to amoxicillin, amoxicillin-clavulanate, ampicillin, ampicillin/sulbactam, first- and second-generation cephalosporins and cephamycins[9,10]. *C. koseri* is inherently resistant to ampicillin, piperacillin and ticarcillin[9,10] Resistance to other b-lactams, through production of extended spectrum b-lactamases, AmpC and carbapenemases , as well as to fluoroquinolones and aminoglycosides has been reported[9]. The resulting PCR products were analyzed by electrophoresis with 1.5% agarose gels in Tris - borate – EDTA buffer. The gels were stained with ethidium bromide and a band was observed at desired position was photographed on an ultraviolet light transilluminator. A molecular weight standard (100 bp ladder-Merck, Bangalore) was included on each gel[11,12]. Mortality rates are as high as 48 to 50%, death is more often associated with polymicrobic than monomicrobic infections[1]. The high mortality rate associated with Citrobacter infections may be due in part to ineffective empirical antibiotic therapy. Citrobacter species are commonly resistant to extended-spectrum cephalosporins[2]. Appropriate and on time treatment of ESBL producing species are given prime importance[2].

Material and Methods

A total of 100 clinical samples from the Various clinical samples received in the central lab of Indira Gandhi institute of medical sciences, Patna, Bihar. Study duration of two years. Identification will be done by using standard microbiological techniques. Antibiotic susceptibility test will be performed by Kirby-Bauer Disc diffusion method. Test strains will be pre-incubated in peptone water at 37ºC at an optical density of 0.5McFarland standard. This suspension will be used to inoculate strains onto Muller Hinton agar plate by swabbing them with a sterile cotton swab and performing culture as recommended by clinical and laboratory standards institute . The antibiotic discs used for sensitivity testing will be obtained from Hi-Media , India , which will be used to study the sensitivity pattern.

Test strains were pre-incubated in peptone water at 37ºC at an opticaldensity of 0.5McFarland standard. This suspension was used to inoculatestrains onto Muller Hinton agarplate by swabbing them with a sterile cotton swab. In this test 30microgram antibiotic disc of ceftazidime , cefotaxime , ceftriaxone and aztreonam are placed on the plate , 30mm from the amoxicillin/clavulanate (20/10 microgram) disc. A clear extension

of the edge of the cephalosporin inhibition zone towards the disk containing clavulanate is interpreted as synergy, indication ESBL production. The British society of Antimicrobial Chemotherapy has recommended the disk diffusionmethod for phenotypic confirmation of ESBL presence using ceftazidime/clavulanate andcefotaxime/clavulanate combination disc with semiconfluent growth on Iso-sensitiser agar. The zone diameter of each combination is compared with zone diameter of cephalosporin along a ratio of cephalosporin/clavulanate zone diameter to cephalosporinzone size is clavulanate. A ratio of >1.5 or greater indicates the presence of ESBL.

Table 1: No of *Citrobacter* isolates in different age groups

AGE GROUP(years)	NO OF ISOLATES (percentage)
0-10	7 (7%)
11-20	4 (4%)
21-30	16 (16%)
31-40	26 (26%)
41-50	17 (17%)
51-60	9 (9%)
61-70	13 (13%)
71-80	5 (5%)
81-90	3 (3%)
TOTAL	100

no of *Citrobacter spp* isolated in different age groups.Maximum isolates were in the age group of 31-40y (26) and least isolates were inthe age group of 81-90y.

Table 2: No of *Citrobacter spp* in different sex

SEX	**NO OF ISOLATES (percentage)**
Males	56 (56%)
Females	44 (44%)
Total	100

no of *Citrobacter spp* isolated in males (56)and females(44).

Table 3: Speciation of *Citrobacter*

Organism isolated	**No of isolates (percentage)**
Citrobacter freundii	18 (18%)
Citrobacter koseri	82 (82%)

Table 4: percentage of ESBL producers in *Citrobacter spp*

ORGANISM	**PERCENTAGE**
ESBL PRODUCERS	62%
NON-ESBL PRODUCERS	38%

information regarding no. Of ESBL producers in *CItrobacter spp*

Discussion

All the samples which have come for the culture and sensitivity testing are taken as test

samplesin this study. From the various departments of the hospital , the samples such as urine, pus , sputum, wound discharge, etc were collected and processing was done accordingly.The observation of this study is as follows...

During this study period, all the 100 *Citrobacter spp* isolates from all the age groups are taken. In this study , maximum *Citrobacter spp* isolates were from 31y-40y i.e., 26% followed by 41y-50y(17%), 21y-30y(16%), 61y-70y(13%), 51y-60y(9%), 0-10y(7%), 71y-80y(5%), 11y-20y(4%) and least number of *Citrobacter spp* are isolated from 81y-90y of age group showing about 3% of total isolates whereas in study conductedby Ritu Nayar, Indu Shukla, Asfia Sultan, maximum number of isolates were from 11- 20y of age i.e., 21.9% and least number of *Citrobacter spp* were isolated from age >60y about 0.95%[1].Similar results are observed in another study done by Hiba Sami, Asfia Sultana, Meher Rizvi et al, which states most of the *Citrobacter spp.* were isolated in the20–30(43.4%) and 30–40 year(17.7%) age group[13]. In contrary , a study done byBasavaraj C Metri, P. Jyothi, Basavaraj V Peerapur, shows 35.5% of isolates are from age>61y followed by 41-60y(25%) and least in 21-40y(17.1%)[9] In the study done by Shobha K.L , Akshatha S.J, Amin Sonam G.S, Out of 160 patients 34 were females (52.3%) and 31 were males (47.69%)[4].In another study done by Hiba Sami, Asfia Sultana, Meher Rizvi *et al* , among the 246 (3.46%) patients positive for Citrobacter spp., 193 were females (78.4%) and 53 males (21.5%)[13].But in our study out of 100 patients whose samples isolated *Citrobacter spp*, there are 56% of males and 44%of female patients. There is increase *Citrobacter* isolates in males more than females. In our study ,most of the *Citrobacter spp* are isolated from urine sample which accountsto 61%, followed by pus sample which were isolated 18%, then sputum -14%, other samples like vaginal swab-4%, nasal discharge-1%, blood-1% and fluid-1%. In a study done by Basavaraj C Metri *et al* 55% of patients *Citrobacter spp* are isolateddue to indwelling urethral catherisation or genitourinary instrumentation or due toobstructive uropathy. The possible reasons for high frequency in elderly male include obstructive uropathy due to prostate enlargement, loss of bactericidal activity of prostaticsecretions frequent genitourinary instrumentation and catheterisation. *Citrobacter koseri*was the predominant organism accounting 72.4% of isolates[14].In a study done by Glenn R. Hodges. M.D. *et al* 37 of 83(45%) suspected infections are due to *Citrobacter* invovled the urinary tract. All the patients were male ranging in age from 23 to 86years. 18 of 37 (49%) episodes in this group were significant infections. 16 of 37(43%) infections were intermediate , and in 3 cases (8%) the isolates were commensals[15]. In another study done by Hiba Sami, Asfia Sultan *et al.* The prevalence of *CItrobacter spp*is 3.46% when compared to other studies which showed 9.4% .Almost half of the patientswere belonged to 21-30 years of age group affected by UTI caused by *Citrobacter spp.*According to our study , the best sensitive drug for treating *Citrobacter* infections is gentamicin (89.7%), Amikacin (83%) , Nitrofurantoin(86.8%) followed by ofloxacin (51.2%) ,Cefotaxime(45%) ,Ceftazidime(37%), Ceftriaxone(35%), Ciprofloxacin(33%), Norfloxacin(24.5%) and Nalidixic acid(19.6%). A study done by Basavaraj C Metri et al, says majority of the UTI s were found to be resistant to Cefotaxime, Cephalaxin, norfloxacin , Ciprofloxacin and the aminoglycosides but since good invitro activity was shown by nitrofurantoin it may be considered as first line oral therapy in ambulatory patients[12].In a study done by Liun Liu, Daoli Chen et al, most of the 82 *C. freundii* isolates were resistant to beta-lactams, especially to penicillins(41.5%), Cephalosporins (19.5%-98.8) and monobactams (25.6%).resistance to two quinolones (ciprofloxacin and levofloxacin) tested was 7.3 and 2.4% respectively.; resistance to other

antibiotics included aminoglycosides (2.4-11.0%), phenicols(2.4%) ,sulphonamides (6.1%) , tetracyclines (8.5%) and nitrofuran(13.4%)[16]. But in a study done by Glenn R. Hodges et al, the drug pattern was little different. Here, more than 90% isolates of *C.freundii* from 21infectious episodes and *C.* 5 isolates showed resistant to all the first line of drugs which were further processed to check sensitivity pattern for second line of drugs. The following is the sensitivity patternof second line of drugs used in the present study : Imipenem(60%), Meropenem(80%), Azithromicin(40%), levofloxacin(60%), Linezolid(0%), Clindamicin(0%) and Piperacillin tazobactum(0%). But in a study done by C Rodrigues e al, ESBL producers in *Citrobacter spp* is accounted to 33.33% where one out of three isolates produced beta-lactamase enzyme[17].In another study done Neena V. Nagdeo, Navinchanda M. Kaore, Vilas R. Thombare, 15 *Citrobacter spp* were isolated and were processed for ESBL detection. Following which 8 isolates were ESBL producers accounting for 53.33% in *Citrobacter* alone[18].There is about 50% incidence of ESBL production among *Citrobacter spp*isolated. *Citrobacter* and similar species may play a unique role in bacterial evolution. They are oflow virulence and thus can persist in a host population for long periods. Over time, they could accumulate resistance determinants which may then be available for transfer to more virulent organisms[19].

Conclusion

The magnitude of *Citrobacter* infections has increased over time considering its potentialto cause nosocomial infections and the growing numbers of immunocompromisedpatients in hospitals. *C.koseri* and *C.freundii* being the commonest species isolated. Theyare usually isolated from patients with wound infections, urinary tract infections , respiratory infections and bacteremia. These are monomicrobial in 83% of cases but polymicrobial infection can also be encountered.*Citrobacter* infection can cause infectionin any age group with significant predilection in adolescent and middle age. Infection isseen in both sexes with significant proportion in males.

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